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1000 units/ml granulocyte and macrophage colony stimulating factor (GM-CSF), 10 µg/ml of interleukin-4 (IL-4), 10% heat inactivated fetal calf serum (FCS), 4mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin sulphate and 100mM β-mercaptoethanol. At day 6 cells showed markers of mature dendritic cells (DCs), and expression of the mannose receptors.

### ***Ex vivo* Treatment of Dendritic Cells with Mannan-Antigen Conjugates**

The mannose receptor-bearing dendritic cells as described above were washed, resuspended in the same culture media at  $1 \times 10^6$  cells/ml. A conjugate of mannan-Cripto, prepared as described in the present application, was loaded on to the DCs for 2 hours by adding the conjugate to the culture medium.

Cripto is a protein expressed in embryonic and cancer cells. It is not expressed in normal tissues. The sequence of CRIPTO used here is a 17-mer peptide that is identical in both human and mouse CRIPTO. The sequence is: CPPSFYGRNCEHDVRKE, and is an antigenic portion of the Cripto protein.

Pulsed DCs were then washed thoroughly, resuspended at  $1 \times 10^7$  cells/0.5ml in PBS (phosphate buffered saline) and 50 µl was injected intradermally in mice in the hind footpads. 10 days later mice were boosted.

### **Antigen Recall in Mice Treated with Pulsed Dendritic Cells**

After 10-14 days, mice were sacrificed and splenocytes were isolated. Antigen recall (to measure the ability of the administered DCs to stimulate the immune response *in vivo* upon infection by an antigen) was assessed by ELISPOT IFN-gamma assays (which is a measure of T cell immune response) after addition of test or control antigens.

The test antigen was the Cripto 17-mer epitope described above. The control antigen was from an epitope comprising the Variable Number of Tandem Repeats (VNTR) of amino acid residues from Mucin1 (Muc1), a protein expressed on various tumour cells. In addition, a positive control consisting of ConA was used.

The results are shown in the attached figures.

As can be seen from the figures, pulsing DCs with a conjugate comprising mannan-Cripto in accordance with the invention described in the present application, and

administration of the pulsed cells to animals, resulted in *in vivo* stimulation of IFN-gamma by the 17-mer Cripto peptide (i.e there is antigen recall). The degree of stimulation was comparable with the response to Con A, the positive control, but substantially higher than the control where immune cells were not exposed to any antigens (indicated by a “-“ under the histogram at the extreme right in each of the figures).

The antigen recall of the 17-mer Cripto peptide was also substantially greater than with VNTR, indicating a specific antigen selection and presentation by APCs, and leading to generation of a T cell immune response to the Cripto peptide *in vivo*.

In conclusion, the results show that the Cripto 17 mer peptide, a cancer antigen that is distinct from Muc1, can stimulate DCs *ex vivo* when conjugated with mannan, enabling the pulsed DCs to stimulate T cell immune response to the antigen *in vivo* following administration.

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Date: \_\_\_\_\_

By: \_\_\_\_\_  
Geoffrey A. Pietersz

